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A Novel Adenovirus–Adeno-Associated Virus Hybrid Vector That Displays Efficient Rescue and Delivery of the AAV Genome

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ABSTRACT

Adenovirus and adeno-associated virus (AAV) are eukaryotic DNA viruses being developed as vectors for human gene therapy. The strengths of each system have been exploited in a novel vector that is based on an adenovirus–AAV hybrid virus incorporated into a plasmid-based molecular conjugate. Efficient rescue and replication of the recombinant AAV genome in this hybrid required transient expression of rep. This feature was incorporated into the transducing particle by conjugating a rep expression plasmid to the hybrid virus through a polylysine bridge. The resulting particle is an attractive vehicle for gene therapy because it is easily manufactured and capable of efficiently transducing cells with the end result being rescue and replication of the recombinant AAV genome. This particle is also useful in the production of recombinant AAV resulting in yields 10-fold greater than that achieved with transfection-based protocols.

OVERVIEW SUMMARY

A novel vector has been developed that represents a hybrid between adenovirus and adeno-associated virus (AAV). This allows for high-level production of vector and efficient gene transfer. The AAV genome is rescued from the hybrid when rep is expressed. An *in vivo* transducing particle that contains all the necessary functions was constructed by chemically conjugating a rep-expressing plasmid to the hybrid virion.

INTRODUCTION

CRITICAL TO THE SUCCESS OF GENE THERAPY is the development of safe and efficient gene transfer vehicles. Limitations in safety and efficiency that exist with current vector systems will substantially restrict their application to human therapies. This shortfall in vector technology is amplified by the ever-increasing spectrum of clinical applications which places more stringent demands on the vector systems. Requisite features of vectors for treatment of chronic diseases include efficient and stable engraftment of the therapeutic gene in a way that can be safely and repeatedly administered. Vectors based on recombinant retroviviruses were used in *ex vivo* applications; however, they have proven to be of little use in most other applications, especially those based on *in vivo* delivery (Miller, 1992). The two functionally related DNA viruses, adenoviruses and adeno-associated viruses (AAV), are being exploited in the development of *in vivo* vector systems (Mulligan, 1993).

Recombinant forms of human adenoviruses have been the subject of intense evaluation for *in vivo* gene therapies (Berkner, 1988; Kozarsky and Wilson, 1993). First-generation vectors, which have been rendered replication defective by deletion of the E1a and E1b genes, have shown great potential in achieving high-level gene transfer to a large variety of cell types *in vivo* including those that are fully differentiated and not dividing. An important advantage is the ease with which one can purify large quantities of highly concentrated recombinant virus. Limitations have been the development of immune responses to the virus and virus-infected cells (Yang *et al.*, 1994, 1995), the latter of which may be overcome by the development of newer recombinants that do not express viral proteins (Engelhardt *et al.*, 1994). Concerns exist over the stability of the recombinant adenoviral genome, which does not integrate or replicate.

AAV is a human parvovirus that is dependent on helper virus such as adenovirus for growth (Carter, 1990). The wild-type

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AAV genome, which exists in the virion as (+) or (−) single-stranded DNA, encodes genes responsible for replication (rep) and formation of virions (cap). Recombinant forms of AAV (rAAV), produced as vectors for gene therapy, have been deleted of all viral open reading frames retaining only the inverted terminal repeat sequences (Muzyczka, 1992; Kotin, 1994). Production of rAAV has been limited to low-yield protocols based on transfection of the rAAV vector with a rep/cap plasmid into cells that are subsequently infected with helper virus (Samulski *et al.*, 1989). The latent phase of the AAV growth cycle, which occurs in the absence of helper virus, leads to efficient integration of a proviral form of its genome to a specific location on chromosome 19 (Kotin *et al.*, 1990; Samulski *et al.*, 1991). The utility of rAAV for gene therapy is based, in part, on its potential to efficiently integrate which, in the absence of rep, is not site specific (Kotin, 1994; Shelling and Smith, 1994; Weitzman *et al.*, 1994).

The early development of adenoviruses and AAV vectors, briefly reviewed above, suggests complementary strengths and weaknesses. Adenoviral vectors efficiently transduce a wide variety of cells (quiescent or dividing) and are easily produced and purified; disadvantages include immunogenicity and instability of the recombinant genome which neither integrates nor replicates. This contrasts with rAAV vectors that are void of all viral open reading frames and can efficiently integrate but cannot be produced in large quantities or be efficiently transduced into nondividing cells. In this report, we describe a novel vector system that takes advantage of both viral systems through the creation of an adenovirus–AAV hybrid. This is further engineered by forming a ternary complex in which a rep expression plasmid is complexed to the hybrid virus through a poly-L-lysine bridge. The resulting hybrid virus–molecular conjugate is easily produced in high quantities and efficiently infects cells, and the rAAV domain is rescued and replicated.

MATERIALS AND METHODS

Hybrid virus construction

The hybrid adenovirus–AAV vector (Ad.AAV) was engineered by homologous recombination in 293 cells as detailed in Fig. 1. Briefly, viral DNA was extracted from CsCl-purified *dl*7001 virions, an Ad5 (serotype subgroup C) variant deleted of 3 kb spanning the nonessential E3 region (Kozarsky and Wilson, 1993). The complementing plasmid, pAd.AV.CMV*lacZ* was constructed by inserting a recombinant AAV genome (AV.CMV*lacZ*) into the *Bgl* II site of the plasmid pAd.BglII. The parental pAd.BglII cloning vector encodes two segments of wild-type Ad5 genome, map units 0–1 and 9–16.1, separated by a unique *Bgl* II cloning site for insertion of heterologous sequences (Kozarsky and Wilson, 1993). The missing Ad5 sequences between the two domains (bp 361–3,327) results in the deletion of E1a and the majority of E1b following recombination with viral DNA. The linear arrangement of AV.CMV*lacZ* includes the 5′ AAV ITR (bp 1–173), obtained by PCR using pAV2 (Laughlin *et al.*, 1983) as template, cytomegalovirus immediate early enhancer/promoter, SV40 splice donor-splice acceptor, *Escherichia coli* β-galactosidase (β-Gal) cDNA, SV40 polyadenylation signal, and 3′ AAV ITR (obtained from pAV2 as a *Sna* BI–*Bgl* II fragment).

Adenoviral DNA was prepared for co-transfection by digestion with *Cla* I (bp position 917), which removes the left arm of the genome encompassing map units 0–2.5. Plasmid DNA was linearized using a unique *Nhe* I site immediately 5′ to map unit zero. The DNAs were delivered to 293 cells using a standard calcium phosphate transfection procedure. Twenty-four hours later, the transfection cocktail was removed and the cells were overlayed with 0.8% agarose containing 1× Basal Medium Eagle (BME) and 2% fetal bovine serum (FBS).

Once viral plaques developed (typically 10–12 days post-transfection), they were removed from the agar overlay with a sterile pasteur pipette and transferred to a cryovial containing 0.5 ml of Dulbecco's modified Eagle's media (DMEM). The suspended plaques were subjected to three rounds of freeze (dry ice/ethanol)–thaw (37°C) and an aliquot was used to infect duplicate plates of fresh 293 monolayers. Twenty-four hours post-infection, cells from one set of plates were fixed and stained for *lacZ* activity as described (Fisher *et al.*, 1996). Cells from the duplicate plate were harvested in 0.5 ml of 10 m*M* Tris-Cl pH 8.0 and lysed by three rounds of freeze–thaw. Cell debris was removed by centrifugation and an aliquot (5 ml) of the supernatant used to measure β-Gal enzyme activity (Kozarsky and Wilson, 1993). Clones identified as positive were taken through two additional rounds of plaque purification. Large-scale production and purification of recombinant virus was performed as described (Kozarsky and Wilson, 1993).

Functional analysis of Ad.AAV hybrids

293 cells were seeded onto six-well 35-mm plates at a density of 1×10^6 cells/well. Twenty-four hours later, seeding media (DMEM/10% FBS supplemented with antibiotics) was replaced with DMEM/2% FBS (1 ml) and infected with putative first-round hybrids. Two hours post-infection, each well was transfected with pRep78/52 (1 μg), a *trans*-acting plasmid that encodes the Rep 78-kD and 52-kD gene products. The rep sequences in this construct are under the control of the AAV P5 promoter and utilize a SV40 polyadenylation signal.

As a positive control for AAV rescue, 293 cells seeded in a six-well plate, as above, were cotransfected with a *cis*-acting AAV plasmid and pRep78/52. The *cis* plasmid, pAV.CMV*lacZ*, contained AV.CMV*lacZ*, the identical sequence encoded by pAd.AV.CMV*lacZ* described above, cloned into the *Bgl* II site of pSP72 (Promega). To provide the necessary adenovirus helper function for AAV rescue, cells were infected with either wild-type Ad5 virus or a first-generation E1/E3-deleted virus H5.CB*ALP* (Kozarsky and Wilson, 1993) at an m.o.i. of 5 approximately 2 hr prior to adding the transfection cocktail.

Transfections were performed with Lipofectamine (Life Technologies) according to the instructions provided by the manufacturer. Cells were harvested 30 hr post-infection and episomal DNA (Hirt extract) was prepared as described (Hirt, 1967). Samples were resolved on a 1.2% agarose gel and electroblotted onto a nylon membrane. Blots were hybridized with a ^{32}P random-primer-labeled restriction fragment isolated from the *E. coli* β-Gal cDNA.

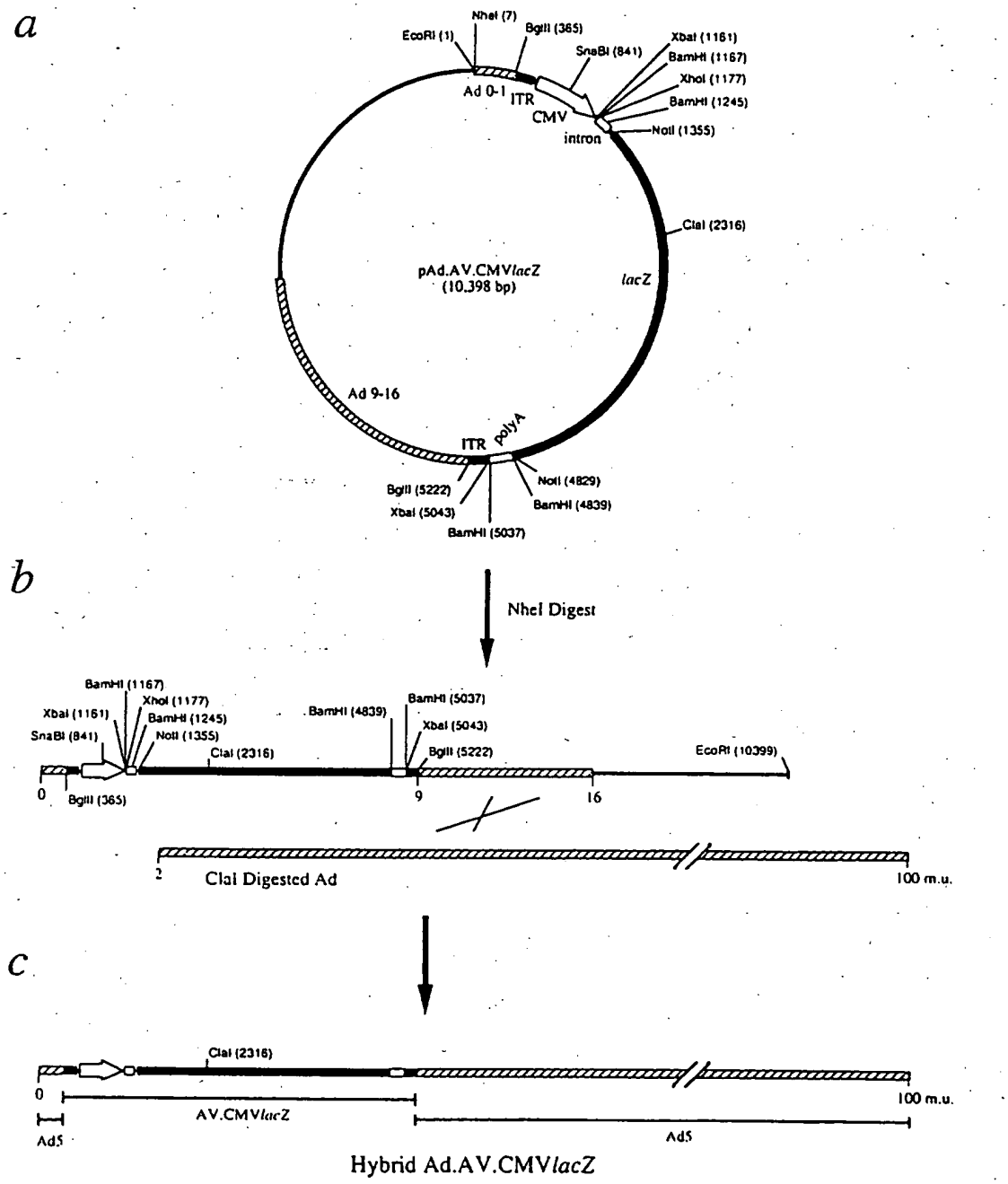


FIG. 1. Strategy for generating a recombinant Ad.AAV virus. a. Organization of pAd.AV.CMV*lacZ*. The labeled viral elements include the 5′ ITR (bp 1–360) from Ad5 (Ad 0–1), AAV ITRs (ITR), and Ad5 sequence spanning map units 9–16 (Ad 9–16). A minigene containing *E. coli* β-Gal (*lacZ*) under the transcription control of a CMV promoter (CMV) is cloned between the AAV ITR sequences. b. The plasmid shuttle vector is linearized with *Nhe* I and transfected along with *Cla* I-digested Ad5 DNA into 293 cells. Homologous recombination occurs between map units 9 and 16 of the plasmid and the corresponding sites in the adenovirus DNA. c. The exchange of sequence between pAd.AV.CMV*lacZ* and Ad5 results in a recombinant adenovirus that now carries a CMV*lacZ* minigene flanked by AAV ITRs. This exchange also results in the deletion of E1 genes.

Synthesis of polylysine conjugates

Purified stocks of Ad.AV.CMV*lacZ* were modified by coupling poly-L-lysine to the virion capsid in a three-step procedure as described (Fisher and Wilson, 1994). Hybrid virions were activated through primary amines on capsid proteins with the cross-linker sulfo-SMCC (Pierce). Unreacted linker was removed by gel filtration through BioGel P-6DG (BioRad).

Poly-L-lysine having a molecular mass of 58 kD (Sigma) as thiolated with 2-imminothiolane (Pierce) to a molar ratio of 2 moles -SH/mole polylysine. Again, unreacted compound was removed by gel filtration. Finally, the two intermediates were reacted on ice for 15 hr under argon. Virus–polylysine conjugates, Ad.AV.CMV*lacZ*-(Lys)$_n$, were separated from free polylysine by ultracentrifugation through a CsCl step gradient with an initial composition of 1.45 gram/ml (bottom step) and 1.2 gram/ml (top step). The final product was dialyzed against 20 m*M* HEPES buffer pH 7.8 containing 150 m*M* NaCl (HBS) and evaluated for DNA-binding capacity by gel-shift assays (Fisher and Wilson, 1994). The DNA-binding capacity is expressed as the number of A_{260} particles Ad.AV.CMV*lacZ*-(Lys)$_n$ that can neutralize the charge contributed by 1 μg of plasmid DNA.

Trans-infection protocol to demonstrate AAV excision and amplification

Ad.AV.CMV*lacZ*-(Lys)$_n$ (6×10^{10} A_{260} particles) in 100 μl of DMEM was added dropwise to a microfuge tube containing 1 μg of plasmid DNA in 100 μl of DMEM. The mixture was gently mixed and allowed to incubate at room temperature for 10–15 min. The *trans*-infection cocktail was added to 293 cells seeded in a 35-mm six-well plate as detailed above. Thirty hours later, cells were harvested and Hirt extracts were prepared. Alternatively, monolayers were fixed 24 hr after addition of the *trans*-infection cocktail and histochemically stained for β-Gal or alkaline phosphatase activity (Fisher and Wilson, 1994). Southern blot analysis of episomal DNA was performed as described above.

Trans-infection protocol for the production of recombinant AAV

Ad.AV.CMV*lacZ*-(Lys)$_n$ conjugate (4.5×10^{13} A_{260} particles) in 75 ml of DMEM was added dropwise with occasional gentle swirling to 75 ml of DMEM containing rep/cap-expressing plasmid (Samulski *et al.*, 1989) and incubated at room temperature for 10–15 min. The complex was diluted with 450 ml of DMEM supplemented with 2% FBS, and 20-ml aliquots were added to monolayers of 293 cells seeded on 150-mm plates. Forty hours post *trans*-infection, cells were harvested, suspended in 10 m*M* Tris-Cl pH 8.0 to a final volume of 20 ml, and stored at −80°C.

Purification of rAAV

Frozen cell suspensions were subjected to three rounds of freeze–thaw cycles to release recombinant AV.CMV*lacZ* and helper hybrid Ad.AV.CMV*lacZ*. On completion of the final thaw, bovine pancreatic DNase (2,000 units) and ribonuclease (0.2 mg/ml final concentration) was added and the extract was incubated at 37°C for 30 min. Cell debris was removed by centrifugation (5,000 × *g* for 10 min) and the clarified supernatant (15 ml) was applied to a 22.5-ml step gradient composed of equal volumes of CsCl at 1.2 gram/ml, 1.36 gram/ml, and 1.45 gram/ml 10 m*M* Tris-Cl pH 8.0. Viral particles were banded at 25,000 rpm in a Beckman SW-28 rotor for 8 hr at 4°C. One-milliliter fractions were collected from the bottom of the tube. A sample (5.0 μl) of each fraction was transferred to a microfuge tube containing 20 μl of capsid digestion buffer (50 m*M* Tris-Cl pH 8.0, 1.0 m*M* EDTA pH 8.0, 0.5% SDS, and 1.0 mg/ml Proteinase K). The reaction was incubated at 50°C for 1 hr, allowed to cool to room temperature, and diluted with milli-Q water (10 μl), and agarose gel loading dye was added. Samples were resolved on a 1.2% agarose gel, electroblotted onto a nylon membrane, and the blot was hybridized with a ^{32}P-labeled *lacZ* fragment as described above.

Peak fractions that contained AV.CMV*lacZ* (r = 1.41 gram/ml) were combined and banded to equilibrium in CsCl using a Beckman NVT-90 rotor (80,000 rpm). The opalescent band of rAAV virus was recovered and subjected to two sequential rounds of equilibrium sedimentation in CsCl using a Beckman SW-41Ti rotor (35,000 rpm). Fractions from the final banding were analyzed for refractive index, absorbance at 260 nm, genome copies (Fisher *et al.*, 1996), and *lacZ* transducing particles. Transduction was measured by infecting monolayers of growing HeLa cells with aliquots (5 μl) from each fraction and staining the cells for *lacZ* activity 24 hr later. Samples were heated at 60°C for 30 min before being added to cells. Titers in *lacZ*-forming units (LFU)/ml were determined by counting the number of blue cells in a field of known surface area and relating that number to the total number of cells that were seeded on the culture dish.

RESULTS

Engineering a recombinant Ad.AAV virus

The first generation of recombinant hybrid vectors was constructed by inserting a proviral clone of *lacZ*-expressing rAAV genome in place of E1a and E1b sequences of Ad5. The synthesis of this recombinant is summarized in Fig. 1. A plasmid called pAd.AVCMV*lacZ* contains Ad5 sequence 0–1 map units and 9–16 map units with an rAAV genome (5′ ITR–CMV promoter–*lacZ* gene–SV40 poly(A)–3′ ITR) in between. This plasmid was co-transfected into 293 cells with *Cla* I-restricted, E3-deleted Ad5 genome, and recombinants were isolated. Recombinant viruses isolated in eight of 10 first-round plaques transduced *lacZ* function and contained the correct structure by restriction enzyme analysis.

The AAV domain can be rescued from the hybrid virus

The ability to rescue the rAAV sequence from the adenovirus shuttle genome is essential to its function as a gene therapy vector. The *in vitro* model used for this study was infection of 293 cells and analysis of Hirt extracts for formation of double-stranded replicative intermediates. Controls for this analysis are presented in Fig. 2a where 293 cells were transfected with rAAV vector and pRep78/52 followed by infection with helper adenovirus. The full spectrum of duplex molecular species that appear during a lytic AAV infection (*i.e.*, monomeric and dimeric forms of the double-stranded intermediates, RFm and RFd, respectively) were evident in transfected cells infected with either wild-type (Fig. 2a, lane + Ad5) or E1-deleted Ad5 (Fig. 2a lane + H5.CBALP). No replicative intermediates were detected when transfections were performed in the absence of helper virus (Fig. 2a, lane − Ad).

Hirt extracts from 293 cells infected with putative Ad.AAV

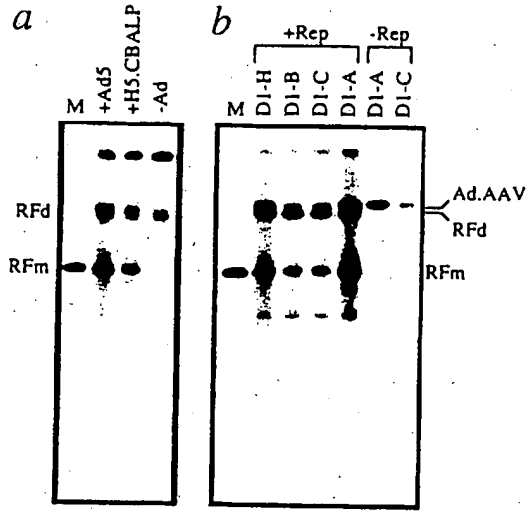


FIG. 2. Screening recombinant Ad.AAV clones for rAAV rescue. a. Hirt extracts from 293 cells infected with wild-type Ad5 (lane +Ad5), an E1-deleted recombinant (lane +H5.CB*ALP*), or not infected (lane −Ad). Two hours post-infection, cells were transfected with a *cis*-acting plasmid containing the AV.CMV*lacZ* genome and a *trans*-acting Rep expression plasmid pRep78/52. b. Hirt extracts from 293 cells infected with putative Ad.AV.CMV*lacZ* hybrid clones were analyzed by Southern hybridization using a *lacZ* probe. Lanes laned +Rep contain samples from cells that were transfected with a Rep expression plasmid 2 hr post-infection. Lanes labeled −Rep contain samples from cells that were transfected with pCMV*ALP* (a plasmid expressing ALP from a CMV promoter) 2 hr post-infection. Above each gel lane is the clone identification. In both a and b, lanes labeled M contain a sample of pAd.AV.CMV*lacZ* (Fig. 1) that was digested with *Bgl* II to release the AV.CMV*lacZ* domain. Duplex monomers (RFm) and dimers (RFd) of the rescued AV.CMV*lacZ* genome, and hybrid Ad.AV.CMV*lacZ* (Ad.AVV) are indicated. These assignments were made based on their predicted sizes and comparisons to molecular weight markers and were run on the same gel.

hybrid clones D1A and D1C revealed a single band when probed with a *lacZ* restriction fragment (Fig. 2b, lanes − Rep) corresponding to the hybrid adenoviral DNA. We reasoned that expression of rep may be necessary for rescue of the rAAV genome from the hybrid. Four independent hybrid isolates, including D1A and D1C described above, were used to infect 293 cells that were expressing rep as a result of transient transfection. Analysis of Hirt extracts revealed the band corresponding to the hybrid DNA in addition to substantial quantities of two other bands whose size corresponds to that of both the RF monomer and dimer of rAAV (Fig. 2b, lanes + Rep).

Rep proteins can be efficiently delivered by an Ad.AAV (Lys)$_n$ conjugate

Although our results indicated the rAAV sequence could be rescued from the hybrid virus, plasmid transfection was required to supply obligatory rep activity, a strategy that is impractical for *in vivo* applications of gene therapy. An approach was developed to deliver the pRep78/52 plasmid directly linked with the hybrid virus via the synthesis of a polylysine conjugate (Fisher and Wilson, 1994). According to the strategy, the rep plasmid is complexed directly to the hybrid capsid through an electrostatic interaction; removing the need for a separate transfection. This single particle contains all functions necessary for the hybrid to function *in vivo* and, therefore, may be useful for *in vivo* applications of gene transfer. Hybrid virions from a large-scale expansion of clone D1A (Fig. 2b) were modified with poly-L-lysine, resulting in an Ad.AAV-(Lys)$_n$ conjugate. Gel-shift analysis indicated the maximal plasmid binding capacity of the purified conjugate was 1 μg of pRep78/52 / 6.0×10^{10} A_{260} particles Ad.AAV-(Lys)$_n$ at a 3:1 molar ratio of plasmid DNA to adenovirual genomes. The resulting complex is called a *trans*-infection particle.

Trans-infection particles were prepared by mixing Ad.AAV-(Lys)$_n$ conjugate with either pRep78/52 or control plasmid DNA prior to application to cells. Hirt extracts of 293 and HeLa cells exposed to these particles revealed a banding pattern consistent with rescue of the rAAV sequence from the parent Ad.AAV virus (Fig. 3a, lanes 293 and HeLa, respectively); both RF monomers and dimers of the rAAV sequence were evident in 293 and HeLa cells. The rescue event was dependent on rep proteins because no AAV RF species were detected in 293 cells exposed to a *trans*-infection particle exposed to an irrelevant reporter plasmid expressing alkaline phosphatase (Fig. 3b, lane

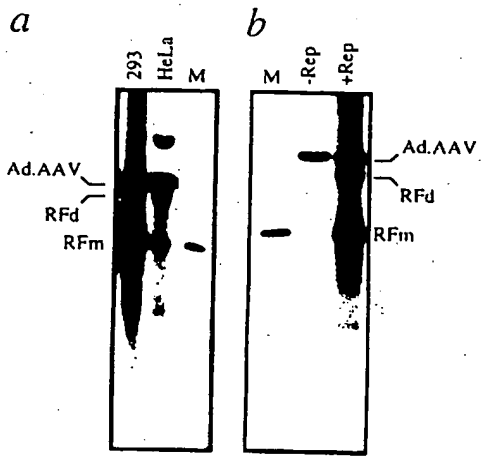


FIG. 3. Rescue of the AAV domain from an Ad.AAV-(pLys)$_n$ conjugte complexed with a Rep expression plasmid. a. Ad.AV.CMF.*lacZ*-(pLys)$_n$ conjugate complexed with a Rep expression plasmid was added to 293 cells (lane labeled 293) or HeLa cells (lane labeled HeLa). Hirt extracts were prepared 24 hr after the addition of vector. b. Ad.AV.CMV*lacZ*-(pLys)$_n$ conjugate was prepared and complexed with a Rep expression plasmid (lane labeled +Rep) or pCMV*ALP* (lane labeled −Rep). The reagent was added to 293, and Hirt extracts were prepared 24 hr later. In both a and b, the blots were probed with a ^{32}P-labeled *lacZ* cDNA fragment. Lanes labeled M contain a sample of pAd.AV.CMV*lacZ* (Fig. 1) that was digested with *Bgl* II to release the AV.CMV*lacZ* domain. Duplex monomers (RFm) and dimers (RFd) of the rescued AV.CMV*lacZ* genome and hybrid Ad.AV.CMV*lacZ* (Ad.AVV) are indicated.

−Rep) that was present when the rep-expressing plasmid was complexed with Ad.AAV virus (Fig. 3b, lane +Rep): A duplicate set of 293 cells that received the conjugate complexed with alkaline phosphatase expression plasmid was histochemically stained for either β-Gal or alkaline phosphatase. Greater than 90% of the monolayer was transduced with both β-Gal (Fig. 4a) and alkaline phosphatase (Fig. 4b) transgenes.

Recombinant AAV can be generated with a hybrid Ad.AAV virus

We next tested whether the rAAV genome that was rescued from the Ad.AAV virus could be packaged into an AAV capsid. A *trans*-infection particle containing the rep and cap-expressing plasmid was incubated with 293 cells. A cell extract was prepared 40 hr later and resolved by buoyant density sedimentation through a discontinuous CsCl gradient. Southern blot analysis of the cell extract before fractionation revealed both hybrid Ad.AAV DNA and double-stranded RF forms (monomers and dimers) of the rescued rAAV sequence (Fig. 5a). A single-stranded monomer of rAAV appeared to be present in the crude extract; however, it was not until the virions were concentrated by ultracentrifugation that the single-stranded genome became clearly evident (Fig. 5b). The single-stranded recombinant genome of the virus was distributed over a range of CsCl densities and displayed a biphasic sedimentation pattern. The two peaks of single stranded rAAV genome occurred at densities of 1.41 and 1.45 g/ml CsCl, consistent with the reported buoyant densities of wild-type AAV in CsCl (de la Maza and Carter, 1980). Analysis of the fractions corresponding to the two vector forms revealed the rAAV-1.41 species was several orders of magnitude more active for *lacZ* transduction than the denser rAAV 1.45 gram/ml variant (data not shown). To avoid confusion with contaminating Ad.AAV, samples were heat inactivated (60°C for 30 min) before incubation with HeLa cells.

The peak fractions of rAAV-1.41 were combined and purified by equilibrium sedimentation in CsCl to eliminate residual adenovirus particles and concentrate rAAV virions. On the final round of ultracentrifugation, a faint but clearly visible opalescent band was observed in the middle of the gradient tube. Fractions that surrounded the band were evaluated for density, absorbance at 260 nm, and *lacZ*-transducing particles (Fig. 6). As the band eluted from the gradient tube, a well-defined peak of 260 nm absorbing material was recorded, with a maximal absorbance occurring at a density of 1.40 gram/ml CsCl (Fig. 6a). Analysis for *lacZ*-transducing particles on HeLa cells revealed a peak of activity (Fig. 6b) that mirrored the absorbance profile (Fig. 6a). The monolayer incubated with fraction #16 is shown in Fig. 6c, in which >80% of the cells were transduced. These results indicate rAAV was efficiently produced from the hybrid Ad.AAV virus. This was most accurately demonstrated by measuring the titer according to genome copies (Fisher *et al.*, 1996); the titer of AV.CMVLacZ using plasmid transfections ranges from 5–10 × 10^{11} genomes/ml, whereas two different preparations generated with the hybrid virus were 5 × 10^{12} and 8.5 × 10^{12} genomes/ml. The propensity to wild-type AAV was no greater using the hybrid system than the traditional transfection based system; wild-type AAV was not present or detected at low quantities (<1 wild-type AAV/10^6 recombinants).

DISCUSSION

Recombinant adenovirus and AAV represent two DNA vector systems that have been engineered for *in vivo* gene transfer. Adenovirus vectors excel in their ability to package relatively large DNAs, grow to high titers, and efficiently transduce a wide variety of both dividing and quiescent cell types (Berkner, 1988; Kozarsky and Wilson, 1993). AAV vectors may be more useful for achieving long-term expression, especially in the context of a stem cell target, because they can integrate (Muzyczka, 1992; Kotin, 1994), although experimental validation of the potential of rAAV for gene therapy is lacking because of difficulties in production and purification of virus. We describe in this report an attempt to combine the strengths of these two systems into a new vector, the Ad.AAV hybrid, with the goal of using adenovirus as a shuttle to deliver an rAAV provirus to the targeted cell.

We focused on the hybrid Ad.AAV system as a gene transfer vehicle *in vivo* and for the high-titer production of AAV. Critical to the success of this strategy for *in vivo* gene transfer was the rescue and replication of the rAAV genome from the parent adenovirus and its persistence as an episome or integrated provirus. *In vitro* studies demonstrated the requirement of rep expression for rescue to occur. One approach for incorporating a rep function into the hybrid was to clone a rep minigene into the adenoviral genome. The well-described inhibitory effects of rep on adenovirus replication made it difficult to isolate this kind of recombinant despite attempts with crippled rep genes and inducible promoters (Casto *et al.*, 1967; Carter *et al.*, 1979). An alternative approach was to incorporate synthetically a rep expression plasmid into the hybrid particle. Poly-L-lysine was directly conjugated to the hybrid virus, which was complexed *via* high-affinity electrostatic interactions to a plasmid that encodes the rep 78 and 52 proteins. The final *trans*-infection particle contains all elements necessary for it to function as an *in vivo* transducing vehicle. Exposure of the particle to the non-E1-expressing cell line HeLa resulted in efficient transduction of the adenoviral genome and rescue and replication of the rAAV domain.

FIG. 5. Production of rAAV. 293 cells were *trans*-infected with Ad.AV.CMV*lacZ*-(pLys)$_n$ complexed to a *trans*-acting AAV plasmid expressing rep and cap. Cells were harvested 45 hr later. a. Low-molecular Hirt DNA from harvested cells. Samples of 1 or 5 μl were loaded onto the gel as indicated. The lane labeled M contains a sample of pAd.AV.CMV*lacZ* (Fig. 1) that was digested with *Bgl* II to release the AV.CMV*lacZ* domain. b. A freeze–thaw extract was prepared and layered onto a discontinuous CsCl gradient. Fractions (1 ml) were collected from the bottom of the centrifuge tube and 5 μl were analyzed by Southern hybridization. Below the panel is a graphic of the centrifuge tube from which the samples were taken. The banding position of infectious hybrid Ad.AV.CMV*lacZ* (Ad.AAV), incomplete hybrid virions (TC), infectious AV.CMV*lacZ* vector (rAAV-1.41), and a denser species of AV.CMV*lacZ* virus (rAAV-1.45) are shown. In both a and b, duplex monomers (RFm) and dimers (RFd) of the rescued AV.CMV*lacZ* genome, and single-stranded progeny (SS) are shown. The band corresponding to hybrid Ad.AV.CMV*lacZ* genome (Ad.AVV) is also indicated.

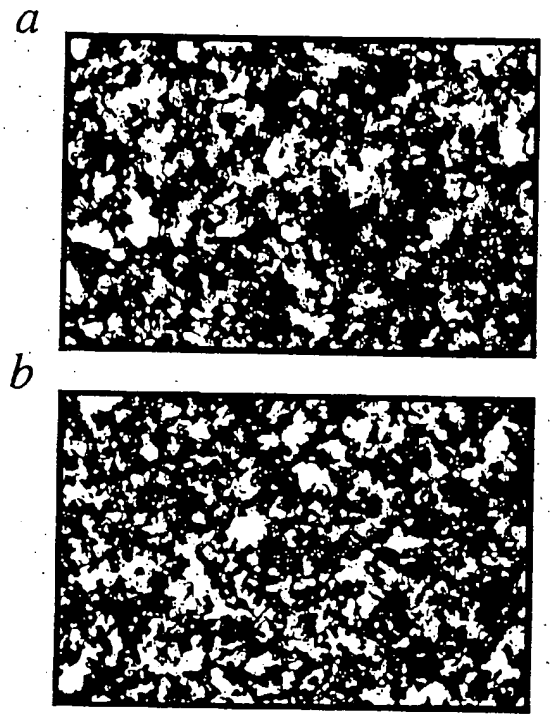
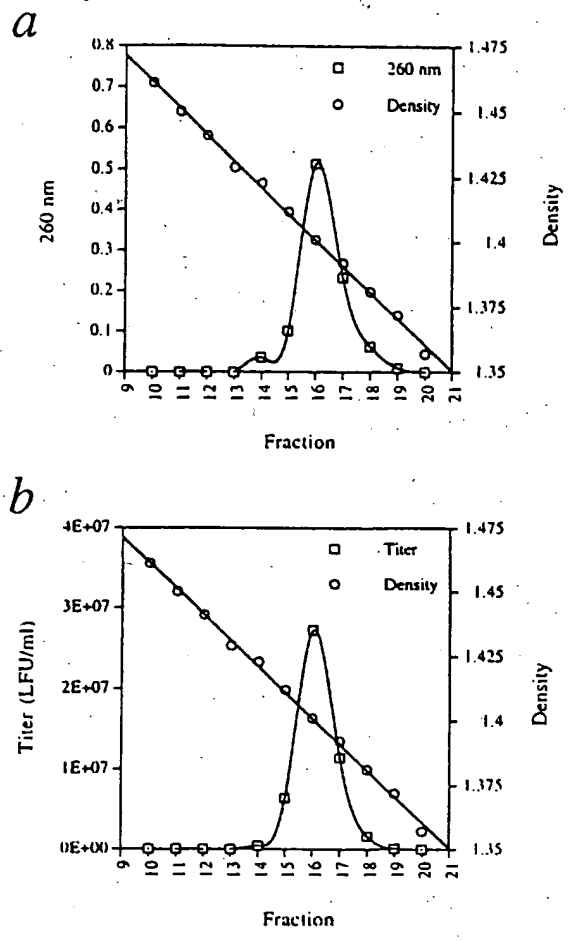


FIG. 4. Histochemical staining for β-Gal and alkaline phosphatase. Control 293 cells that were *trans*-infected with Ad.AV.CMV*lacZ*-(pLys)$_n$ conjugate complexed with pCMV*ALP* (Fig. 4) were fixed and stained for β-Gal activity (a) or alkaline phosphatase (b) activity.



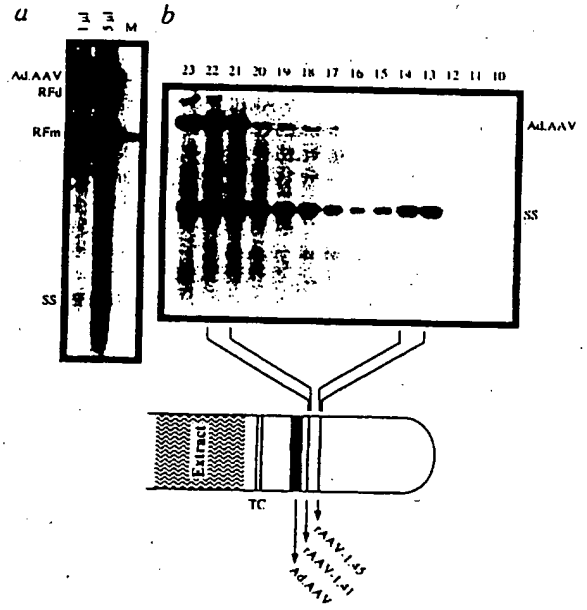


FIG. 6. rAAV transduction. Fractions 18–22 shown in Fig. 5 were combined and purified by three sequential rounds of buoyant density equilibrium sedimentation in CsCl. Fractions that surrounded the opalescent AV.CMV*lacZ* band were analyzed for density, absorbance at 260 nm, and the titer of *lacZ*-transducing particles. a. Absorbance at 260 nm and density are plotted along the vertical axis, whereas fraction number is given on the horizontal axis. b. Titer in LFU/ml and density are plotted along the vertical axis, whereas fraction number is given along the horizontal axis. c. Approximately 1×10^9 A_{260} particles of purified, heat-treated AV.CMV*lacZ* virus from fraction #16 was added to a growing monolayer of HeLa cells. Twenty-four hours later, the cells were fixed and histochemically stained for *lacZ* activity.

The Ad.AAV hybrid virus was also shown to be useful for the production of rAAV. The benefit of incorporating the hybrid Ad.AAV virus into the procedure is that the *cis* AAV element is contained within the parental adenovirus genome, which provides all helper functions. As a result, the *trans* AAV plasmid is the only DNA that requires transfection. Incorporation of the *trans* pRep78/52 plasmid into the hybrid virus *via* the polylysine bridge brings into one particle all *cis* and *trans* functions necessary to produce rAAV. Using the hybrid virus, we have been able to increase our yield of rAAV-transducing particles by 5- to 10-fold.

The *trans*-infection particle initially was developed for *in vivo* gene therapy, in part due to the limitations of rAAV in terms of packaging capacity, production, and efficiency of gene transfer (Muzyczka, 1992; Kotin, 1994). The use of recombinant adenovirus as a delivery vehicle overcomes many of these limitations. Several important questions remain regarding the utility of this approach for *in vivo* gene transfer, especially for the treatment of chronic diseases such as cystic fibrosis where stable expression in nondividing cells is desired. Will the rescued and replicated rAAV genome persist as an episome or integrate? Will integration of the rescued rAAV genome occur with high efficiency and in a site-specific manner? The use of adenovirus as a shuttle vehicle will be associated with the attendant problems of cellular immune-mediated clearance of transduced cells and/or humoral immune responses to the vehicle that precludes a second administration (Yang *et al.*, 1994, 1995). Strategies are underway to prevent cellular responses with improved adenoviruses vectors and to avoid humoral responses with transient immune-blockade (Engelhardt *et al.*, 1994; Yang *et al.*, 1995).

The greatest potential of the *trans*-infection particle may be in *ex vivo* gene therapy where transduction and proviral integration in a stem cell is desired such as in bone marrow-directed gene therapy (LaFace *et al.*, 1988; Walsh *et al.*, 1992; Miller *et al.*, 1994). The use of rAAV has been touted as an alternative to recombinant retroviruses for hematopoietic stem cell gene transfer because of the ability of AAV to transduce nondividing cells and efficiently integrate into a specific site of chromosome (Kotin *et al.*, 1990; Podsakoff *et al.*, 1994). Recent experiments indicate transduction of rAAV into nondividing cells is poor in the absence of rep and, when integration does occur, it does so in a random fashion (Russell *et al.*, 1994). The *trans*-infection particle provides several advantages over rAAV alone: (i) the adenovirus carrier can be grown to titers sufficient for high m.o.i. infections of a large number of cells, (ii) the adenoviral genome is efficiently transported to the nucleus in nondividing cells as a complex facilitating transduction into mitotically quiescent cells, and (iii) incorporation of the rep plasmid into the *trans*-infection particle provides high but transient expression of rep that is necessary for both rescue of rAAV DNA and efficient and site-specific integration (Shelling and Smith, 1994; Weitzman *et al.*, 1994).

The vector system described in this report illustrates a strategy for developing novel gene therapy vehicles based on the formation of a chimera that incorporates features of existing viral- and nonviral-based vector systems. The current strategy utilizes components of two DNA vectors, adenoviruses and AAV, coupled to the versatile molecular conjugate strategy. The resulting *trans*-infection particles are being evaluated in both *ex vivo* and *in vivo* applications of gene therapy.

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